Pathogen Derived Resistance and Reducing the Potential to Select Viruses with Increased Virulence

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INTRODUCTION

Pathogen derived resistance (PDR) refers to using sequences from a pathogen to protect the host from the effects of the pathogen (Sanford and Johnson, 1985). Following the first example of coat protein (CP)-mediated resistance, a type of PDR, to protect transgenic tobacco plants from infection by tobacco mosaic virus (TMV) (Powell-Abel et al., 1986) there have been many reports of different types of PDR. Genes that produce virus-derived antisense RNAs, (+) sense RNAs that do not encode proteins, modified and wild type replicases, and wild type and mutant cell-cell movement proteins have been used to confer resistance. Certain gene strategies appear to be more effective than others depending upon the virus and host. Likewise, certain strategies are anticipated to be more useful in agricultural settings than others. To date, the most common type of PDR in advanced stages of development is CP-mediated resistance; furthermore, government regulatory agencies have approved the use of CP genes to protect plants in agriculture. Other types of genes are being reviewed for similar status.

During the technical stage of development and applications of PDR, a variety of questions have been raised related to the relative safety of transgenic plants that contain virus-derived gene sequences. Some of the concerns are based upon lack of understanding of the methods used to develop resistance, or the biology of virus infection and disease, while certain concerns have some degree of validity. This short paper highlights several of the arguments offered to restrict the use of PDR and those presented to promote its use. This is followed by a summary of recent results that show that knowledge of mechanisms of resistance can lead to increased efficacy of PDR and can reduce concerns about safety and durability of PDR in agriculture.

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STRATEGIES FOR PATHOGEN DERIVED RESISTANCE AND REDUCING BIOSAFETY ISSUES

A) Replicase-*mediated* and *RNA-mediated resistance.* The first report of resistance in transgenic plants that resulted from expression of virus gene sequences involved in virus replication (e.g. replicase, helicase and methyl-transferase) was reported by Golemboski et al. (1990). In this work, the sequence of the TMV 54-kDa open-reading frame conferred high levels of resistance to TMV in transgenic tobacco plants. Subsequent papers reported resistance that was conferred by genes that produce complete, or partial, virus proteins that were either wild type or mutated to eliminate function of the replication protein (Beachy, 1997; Palukaitis and Zaitlin, 1997). In some cases, resistance was due to what is referred to as RNA-mediated resistance (Beachy, 1997). In some examples of replicase-mediated resistance, transgenic plants are highly resistant to infection, and in some cases, are essentially not infected by the virus from which the replicase gene is derived. However, such plants are usually susceptible to closely related viruses and virus strains (Palukaitis and Zaitlin, 1997).

The objection most often raised in relation to use of replicase-mediated resistance is based upon the possibility that recombination may occur between the virus that infects the transgenic plants and the mRNA produced by the transgene. In this situation, mRNA sequences produced by the transgene are 'captured' during replication by the second virus. Capture may occur if replicase jumps, or switches, from the viral genome to mRNA (template switching) produced by the transgene. Template switching may result in chimeric viral RNA that increases the host range or virulence of the resulting virus. Template switching has been observed to occur in plants infected by several viruses that are closely related to each other (Simon and Bujarski, 1994). Transgene caputure was detected in transgenic plants that were challenge-infected by a mutant virus that would survive only if a recombinant virus was produced (Greene and Allison, 1994; Allison et al., 1996). In another case, recombination was observed under conditions that are considered to be less stringent to the selection of recombinant virus (Wintermantel and Schoelz, 1996; Király et al., 1998).

It is now known that template switching or other types of intergenomic recombination occurs more frequently with certain groups of viruses than with others (Simon and Bujarski, 1994). For example, it has been documented to occur with certain potyviruses (Cervera et al., 1993), tobravirus (Robinson et al., 1987; Angenent et al., 1989), bromoviruses (Allison et al., 1989) and geminiviruses (Liu et al., 1998), but apparently occurs at much lower frequency for other viruses (e.g. tobamoviruses) (Fraile et al., 1997).

In considering the potential impact of recombination between viruses and host mRNAs, it is important to recall the biological nature of virus infection. Viruses survive in nature because their replication is ensured by their genetic composition, and viruses that cannot replicate cannot survive. Thus, the likelihood that a virus that lacks a replicase would cause an infection in transgenic plants that contains a replicase gene sequence, and subsequently acquire a functional replicase gene, is very remote. It is theoretically possible (though unlikely) that defective viruses that lack a replicase would be co-transferred during insect or other transmission from plants that are infected by multiple viruses. Such virus may, under very selective and as yet unknown conditions, gain competitive advantage for recombination. It is important to gain a more complete understanding of virus replication and recombination, and, if possible, to reduce or eliminate the likelihood of recombination in pathogen derived resistance. For example, if the "hot spots" for virus recombination are known, it may be possible to develop transgenes that lack such sequences. Indeed, when sequences that are involved in strand switching/ recombination were removed from a transgene, the frequency of sequence capture by challenge virus was substantially reduced or eliminated (Greene and Allison, 1996; Allison et al., 1996). Similar results were found when the sequences involved in recombination between RNAs associated with turnip crinkle virus (TCV) were modified (Cascone et al., 1993).

B) *Movement protein (MP)-mediated resistance.* Cell-cell and long distance spread of virus infection requires one or more viral proteins (Hull, 1991; Séron and Haenni, 1996; Nelson and van Bel, 1998). In certain cases virus host range and pathogenicity are determined by movement protein(s). Therefore, there is legitimate concern about the use of movement proteins to confer pathogen-derived resistance. On the other hand, because many viruses accomplish spread in the host via common, intercellular channels known as plasmodesmata, there is a strong likelihood that durable and broad resistance to multiple viruses could be achieved if cell-cell and long distance spread could be blocked by expression of a transgene (Deom et al., 1992). Several research groups have demonstrated moderate levels of virus resistance in transgenic plants that express non-functional mutants of movement proteins. Such proteins presumably act as dominant negative mutants for one or more functions of movement proteins that are produced during virus infection [i.e. defective movement protein of TMV expressed in transgenic plants confers protection against multiple viruses from different groups (Cooper et al., 1995)].

Based upon the role of movement proteins in virus disease it is easy to understand the concerns about using wild type movement proteins as resistance genes in transgenic plants. However, the fact that sequences of movement proteins are vastly different between different viruses dramatically lowers the likelihood that movement protein sequences will be captured during infection by non-related viruses. Such differences make it highly unlikely that 'functional

domains' could be reconstructed in the event that MP sequences are captured by virus infection. Using movement proteins that are made non-functional by directed mutagenesis further reduces potential risk.

C) *Coat Protein (CP) mediated resistance.* Coat, or capsid proteins, protect the viral nucleic acid from degradation. CP can also be important in several stages in virus infection, including acquisition and transmission of virus by insect vectors, cell-cell and long distance spread in the host, and for some viruses, CP regulates one or more steps of virus replication. It has been argued that transgenic plants that exhibit CP-mediated resistances represent a biosafety risk because the CP may encapsidate the genome of unrelated viruses that infect the transgenic plant, and that such viruses may be acquired and spread to non-hosts by the insect vector. In addition, it is known that certain CPs can encapsidate non-related RNAs (Robinson, 1996). In mixed infections of zucchini yellow mosaic potyviruses strain ZYMV-NAT (non-aphid-transmissible) with papaya ringspot potyvirus (aphid transmissible) their CP molecules can co-assemble. As a result, aphids can acquire and transmit ZYMV-NAT by virtue of mixed encapsidation by both CPs (Bourdin and Lecoq, 1991).

Likewise, it was shown that in transgenic plants heterologous encapsidation of RNA of the challenge virus by the transgenic CP could occur. For example, in transgenic plants that contain CP of the strain N of Potato virus Y (PVY^N), and infected by strain O of PVY (PVY^O), the transgenic CP can co-assemble with the challenge virus (Farinelli et al., 1992). Similarly, it was shown that transgenic plants that contain CP of the aphid transmissible plum pox potyvirus (PPV), and infected by the non-aphid-transmissible strain NAT of ZYMV, transgenic CP could co-assemble with the challenge virus. As a result of the co-assembly, ZYMV can be transmitted by aphids (Lecoq et al., 1993).

DOES *TRANS*-ENCAPSIDATION REPRESENT A SIGNIFICANT RISK TO THE ENVIRONMENT?

Since virus replication is determined by the viral genome rather than the capsid, it is unlikely that *trans*-encapsidation *per se* will lead to permanently expanded host range of a particular virus. Furthermore, since multiple viruses infect many plants, including viruses that are in the same taxonomic group, it is likely that *trans*-encapsidation that may occur in transgenic plants poses no greater risk than *trans*-encapsidation that occurs in mixed virus infections.

It is possible to reduce the risk of *trans*-encapsidation by using CP that is incapable of assembly or insect transmission. Recently, we constructed mutants of TMV CP that are incapable of forming viable virus particles but confer CP-mediated resistance against TMV (Clark et al., 1995; Bendahmane et al., 1997). Similarly, the amino acid sequences on CP molecules that are required for insect acquisition and/or transmission are known for potyviruses (Atreya et al., 1990; Gal-on et al., 1992; Blanc et al., 1997) and cucumber mosaic cucumovirus (Perry et al., 1998). As such sequences are identified they can be removed from

CP genes that are used for CP-mediated resistance (Stark and Beachy, 1989; Jacquet et al., 1998). Taking steps to reduce insect acquisition and virus assembly of CP molecules used in CP-mediated resistance will reduce some of the concerns regarding possible insect transmission of *trans*-encapsidated virus.

Improving the Efficacy of Coat Protein-Mediated Resistance

Based on a series of studies, we proposed that CP-mediated resistance to TMV resulted when CP in the transgenic plant formed an appropriate interaction with CP of the challenge virus to prevent disassembly and virus replication (reviewed by Fitchen and Beachy, 1993). In more recent studies to clarify the molecular mechanisms of CP-mediated resistance, we developed mutants of TMV CP, determined the effect of the mutation on virus assembly, and, whether or not transgenic plants that produced the mutant CPs were resistant to TMV.

Mutants were created, based upon the known structure of the CP and the virus, that were predicted to increase or decrease interactions between CP molecules. In published studies, changes were made to CP codons 28 to encode isoleucine rather than threonine (Clark et al, 1995) or CP codon 28, 42, and 89, to encode tryptophan rather than threonine (Bendahmane et al., 1997). In these cases the volume of the amino acid side chains was increased. None of the mutant CP molecules was capable of assembly with viral RNA, and the proteins were therefore unable to form infectious virus. However, all but one of the mutants was capable of self-assembly and formed various types of aggregates, some of which were virus-like while other mutants produced paracrystalline arrays of CP. Mutant CP that failed to assemble (i.e., did not form virus-like particles or other aggregates) did not confer CP-mediated resistance. Mutants that produced highly stable aggregates conferred high levels of CP-mediated resistance while aggregates with intermediate levels of stability conferred intermediate levels of resistance. Interestingly, the CP mutants that formed virus-like particles that were more stable than those produced by wild type CP conferred a higher level of CP-mediated resistance than did the wild type (Bendahmane et al., 1997). We suggest that increased resistance resulted from increased H-bonds between transgenic CP and challenge virus, which decreased virus disassembly and virus infection.

Conclusions

Pathogen derived resistance can be used to develop transgenic plants that are resistant to virus infection. It is possible to reduce both potential and perceived risks associated with the transgenes by constructing transgenes that reduce potential for trans-capsidation, insect transmission, and sequence capture (recombination). The recent studies of TMV coat protein that resulted in high levels of CP-mediated resistance demonstrate that knowledge of the structural and cellular mechanisms of resistance can lead to the development of a 'second generation' of transgenes that have both increased efficacy and greater environmental safely.

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